

AMENDMENTS TO THE SPECIFICATION

The following is a list of instructions, submitted in accordance with 37 C.F.R. § 1.121(b)(1), to replace certain paragraphs of the specification as-filed with the replacement paragraphs provided herein. As required, each paragraph to be replaced is unambiguously identified and the full text of each replacement paragraph is submitted with markings to show all the changes relative to the previous version of the paragraph.

In the specification as-filed, please replace the paragraphs on page 7 at lines 7 through 21 with the following paragraphs, showing deletions and insertions:

-- In a preferred further embodiment, step (d) is performed by means of the ~~guanidination~~ guanidination of the ϵ -amino group of a lysine residue of a peptide by chemical reaction of peptides with O-methyl iso-urea-hemisulfate, and the ~~nicotinylation~~ nicotinylation of the α -amino group by chemical reaction of peptides with $^2\text{D}_4$ -nicotinyl-amino-hydroxy-succinimide ($^2\text{D}_4$ -NicNHS). The ~~guanidination~~ guanidination of the ϵ -amino group of a lysine residue of peptides is, for example, described in Beardsley et al., 2002, Optimization of ~~guanidination~~ guanidination procedures for MALDI mass mapping, Anal. Chem. 74:1884-1890. The ~~nicotinylation~~ nicotinylation of the α -amino group of peptides is, for example, described in Munchbach et al., 2000, Quantitation and facilitated de novo sequencing of proteins by isotopic N-terminal labelling of peptides with a fragmentation-directing moiety, Anal. Chem. 72:4047-4057.

In a preferred further embodiment, step (e) is performed by means of the ~~guanidination~~ guanidination of the ϵ -amino group of a lysine residue of a peptide by chemical reaction of peptides with O-methyl iso-urea-hemisulfate, and the ~~nicotinylation~~ nicotinylation of the α -amino group by

chemical reaction of peptides with $^1\text{H}_4$ -nicotinyl-amino-hydroxy-succinimide ($^1\text{H}_4$ -NicNHS). --

In the specification as-filed, please replace the paragraphs on page 12 at lines 11 through 33 with the following paragraphs, showing deletions and insertions:

-- FIG. 3 shows a comparative quantification of antigenic peptides from two different sources, wherein in (A) a mass spectroscopic analysis of the relative amount ratios of three different peptides from two tissue samples (colon cancer sample, sample of healthy tissue from the same patient) is shown. The peptides isolated from the colon cancer sample were $^2\text{D}_3$ -acetylated. The peptides isolated from the sample of healthy tissue were $^1\text{H}_3$ -acetylated. (B) shows a mass spectroscopic analysis of three different peptides from $^1\text{H}_4$ -~~nicotinylated/guanidinated~~ nicotinylated/guanidinated Awells-cells, and Awells-cells transfected with keratin 18, and $^2\text{D}_4$ -~~nicotinylated/guanidinated~~ nicotinylated/guanidinated Awells-cells. (C) shows the determination of the amino acid sequences of an $^1\text{H}_3$ -acetylated peptide with the amino acid sequence DAAHPTNVQR and of a $^2\text{D}_3$ -acetylated peptide with the amino acid sequence DAAHPTNVQR by fragmentation;

FIG. 4 shows yields of peptides that have been chemically modified in four different ways. Four peptides with the amino acid sequences AETSYVKVL, KLSLGLPGL, SLGLQLAKV and VLDPRGIYL were used in a mixture in equimolar amounts, and were subsequently for the purpose of the comparative examination of the three strategies for chemical modification either acetylated, or acetylated and ~~guanidinated~~ guanidinated, or ~~guanidinated~~ guanidinated and ~~nicotinylated~~ nicotinylated. After finalisation

of the chemical reaction for modification of the reference peptides, these were mixed with the initially used non-modified peptides in order to allow for a comparison in the following analytic step. The comparative evaluation was performed by analysis with nano-electrospray-ionisation-mass spectrometry (nano-ESI-MS). --

In the specification as-filed, please replace the paragraph on page 14 at lines 6 through 28 with the following paragraph, showing deletions and insertions:

-- ~~Guanidination~~ Guanidination of Peptides

To a peptide mixture from tumour tissue (CCA129), or keratin-18-transfected or non-transfected Awells-cells (amounts of peptides in the mixtures: between 2 nmole and 200 pmole) in citrate-buffer (50 mM citrate, pH 3.0) 0.25% trifluoro-acetic acid (TFA, per volume) was added, subsequently the pH of the mixture was adjusted with 200 µl sodium hydroxide (10 M solution) to 10.5. After addition of 1 ml O-methyl-iso-urea-hemisulfate-solution (2.5 M in water), the reaction mixture was incubated for 10 minutes at 65°C. (water bath). The reaction was stopped by the addition of 200 µl formic acid.

~~Nicotination~~ Nicotinylation of ~~Guanidated~~
Guanidinated Peptides

The peptide mixture from tumour tissue (CCA129) that was chemically modified by ~~guanidination~~ guanidination, or keratin-18-transfected or non-transfected Awells-cells were loaded on a chromatographic column of the type "reversed phase C-18 microcolumn" (Agilent Technologies hydrophobic XGSXB), and washed with 0.5 ml water. The peptide that was bound to the material of the column was

then left on the column, and was ~~nicotinized~~ nicotinylated by chemical reaction at room temperature by slowly adding 1 ml of freshly prepared $^1\text{H}_4$ - or $^2\text{D}_4$ -~~nicotinyl~~ nicotinyl-N-hydroxysuccinimide-ester (sodium phosphate buffer 50 mM; pH 8.5). Following this, for a second time 1 ml of freshly prepared $^1\text{H}_4$ - or $^2\text{D}_4$ -~~nicotinyl~~ nicotinyl-N-hydroxysuccinimide-ester is slowly applied through the chromatographic column that is loaded with the peptide mixture. Then, hydroxylamine was applied through the column in order to remove again unwanted modifications of tyrosine residues by nicotinyl groups. Finally, the chromatographic column was washed with water, before the peptides were eluted from the column with 100 μl of a 50% acetonitrile/water-mixture (per volume). --

In the specification as-filed, please replace the paragraphs from page 19 at line 25 to page 21 at line 6 with the following paragraphs, showing deletions and insertions:

-- The first-time and novel combination of two methods for chemical modification of peptides by combination of the uniform ~~guanidination~~ guanidination of ϵ -amino group of lysine residues in peptides by O-methyl-isourea-hemisulfate, and the ~~nicotination~~ nicotinylation of the α -amino group the peptides by NicNHS resulted in a clear improvement of the ionisation of peptides (FIG. 4). In order to simplify the desalting of the chemically modified peptides, the ~~nicotination~~ nicotinylation of the peptides is performed as described on a C18-chromatography column. The unwanted modification of the side chains of tyrosine residues caused by the ~~nicotination~~ nicotinylation could be removed again by treatment of the modified peptides with hydroxylamine. As an example FIG. 4 shows, based on the peptide having the amino acid sequence AETSYVKL, that the ~~nicotination~~

nicotinylation of the N-terminus influences the ionisation in way that results in a detection of ~~nicotinated~~ nicotinylated peptides that is as good as with chemically non-modified peptides.

Identifying and Quantifying of MHC-Class-I-Bound Peptides from the Awells Cell Line and the Awells Cell Line Transfected with a Plasmid Containing the cDNA of the Human Keratin 18 by ~~Guanidination~~ Guanidination and $^2\text{D}_4$ -/ $^1\text{H}_4$ -~~Nicotination~~ Nicotinylation of the Peptides.

It was shown by Trask et al., 1990, Keratins as markers that distinguish normal and tumour-derived mammary epithelial cells, Proc. Natl. Acad. Sci. U.S.A., 87:2319-2323, that ~~keratines~~ keratins are suitable as markers for distinguishing between tumour and healthy tissue. In order to identify novel MHC-class-I-bound peptides from human keratin 18, and in order to show the differential quantifying based on ~~[[a]]~~ an exemplary tumour antigen, peptides were isolated from the non-transfected (Awells) and from the Awells-cell line (Awells Keratin 18) transfected with the plasmid as given. Subsequently, the isolated peptide-mixture was chemically modified as described by ~~guanidination~~ guanidination and $^2\text{D}_3$ -, and $^1\text{H}_3$ -~~nicotination~~ nicotinylation, respectively. The chemically modified peptide mixtures were mixed, and examined by HPLC-MS-analysis as described. A second experiment was performed in the MSMS-modus, whereby the amino acid sequences of a total of 27 different peptides could be determined. All 27 peptides as found, with the exception of one peptide having a molecular weight of ~~4091,6~~ 1091.6 Da, were detected both in transfected as well as on non-transfected cells in amounts that were located within the confidence interval of 0.64 to 2.28 (statistic

evaluation by using the “student’s t-test”). For the peptide with a molecular weight of ~~4091.6~~ 1091.6 Da, the amino acid sequence RLASYLDRV was determined by the MSMS-analysis, which represents a fragment of the amino acid sequence of keratin 18. The MSMS-spectra that led to the identification of the peptide with the sequence RLASYLDRV are shown in FIG. 3D. For the peptide RLASYLDRV, no signal could be detected that could be correlated to a chemical modification of the primary sequence with a $^1\text{H}_3$ -nicotinyl residue. This observation leads to the assumption that keratin 18 was exclusively expressed in the Awells keratin 18-cells. In contrast, the signal for the peptide RLASYLDRV with a $^2\text{D}_3$ -nicotinyl residue was expressed six fold higher compared to background.

The described methods of the ~~guanidination~~ guanidination and ~~nicotination~~ nicotinylation of peptides by using the two hydrogen isotopes ^1H and ^2D for the first time allows for the rapid and exact determination of relative quantitative differences between peptides having the identical sequence from two or more different sources. By use of the methods for ~~guanidination~~ guanidination and ~~nicotination~~ nicotinylation of peptides on samples of tumour tissue and normal tissue of the same organ, or by use of said methods on cell lines that were transfected prior with nucleic acids encoding for oncogenes or other tumour-associated gene products, tumour-associated peptide antigens can be determined that are particularly suitable for the production of vaccines for cancer therapy. --